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<b>(21) International Application Number:</b> PCT/US91/01282 <b>(22) International Filing Date:</b> 28 February 1991 (28.02.91)  <b>(30) Priority data:</b> 488,608                      2 March 1990 (02.03.90)      US 537,430                      13 June 1990 (13.06.90)      US  <b>(71)(72) Applicant and Inventor:</b> WILLIAMS, Diane [US/US]; 22 Danforth Way, Franklin, MA 02038 (US).  <b>(74) Agent:</b> CLARK, Paul, T.; Fish & Richardson, 225 Franklin Street, Boston, MA 02110-2804 (US).  <b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), BG, CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, LU (European patent), NL (European patent), NO, PL, RO, SE (European patent), SU.		<b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> IMPROVED CHIMERIC TOXINS  <b>(57) Abstract</b>  A chimeric toxin comprising protein fragments joined together by peptide bonds, the chimeric toxin comprising, in sequential order, beginning at the amino terminal end of the chimeric toxin, (a) the enzymatically active Fragment A of diphtheria toxin, (b) a first fragment including the cleavage domain 1 <sub>1</sub> adjacent the Fragment A of diphtheria toxin, (c) a second fragment comprising at least a portion of the hydrophobic transmembrane region of Fragment B of diphtheria toxin, the second fragment having a deletion of at least 50 diphtheria toxin amino acid residues, the deletion being C-terminal to the portion of the transmembrane region, and the second fragment not including domain 1 <sub>2</sub> , and (d) a third fragment comprising a portion of a cell-specific polypeptide ligand, the portion including at least a portion of the binding domain of the polypeptide ligand, the portion of the binding domain being effective to cause the chimeric toxin to bind selectively to a predetermined class of cells to be attacked by the enzymatically active Fragment A.		

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## IMPROVED CHIMERIC TOXINS

### Background of the Invention

This application is a continuation-in-part of  
USSN 488,608 filed March 2, 1990.

This invention relates to the use of  
5 recombinant DNA techniques to construct chimeric toxin  
molecules.

The literature contains many examples of fused  
genes which code for chimeric proteins. For example,  
Villa-Komaroff et al. (1978) Proc. Natl. Acad. Sci.  
10 U.S.A. 75:3727-3731, describes a fused gene made up of a  
eukaryotic structural gene fused to a non-cytoplasmic  
bacterial gene. The fused gene codes for a chimeric  
protein which is transported out of the cytoplasm.  
Murphy U.S. Patent No. 4,675,382, hereby incorporated by  
15 reference, describes the use of recombinant DNA  
techniques to produce a hybrid, or chimeric, protein,  
consisting of a portion of the diphtheria toxin (DT)  
molecule linked via a peptide linkage to a cell-specific  
ligand such as  $\alpha$ -melanocyte stimulating hormone  
20 (MSH). The DT-MSH chimeric toxin was selectively toxic  
for particular target cells, i.e.,  $\alpha$ -MSH receptor  
positive human malignant melanoma cells.

A diphtheria toxin-related fusion protein,  
DAB<sub>486</sub>-IL-2, in which the native receptor binding  
25 domain of DT was genetically replaced with a portion of  
the polypeptide hormone interleukin-2 (IL-2) has been  
described in Williams et al. (1987) Protein Engineering  
1:493-498, hereby incorporated by reference.  
DAB<sub>486</sub>-IL-2 is a 68,142 Da fusion protein consisting  
30 of, in the following order: Met; DT residues 1-485; and

amino acids 2 through 133 of mature human IL-2.

DAB<sub>486</sub>-IL-2 has been shown to bind

to the IL-2 receptor and to selectively intoxicate lymphocytes which bear the high affinity form of the

5 IL-2 receptor, Bacha et al. (1988) J. Exp. Med

167:612-622. Moreover, the cytotoxic action of

DAB<sub>486</sub>-IL-2, like that of native diphtheria toxin,

requires receptor-mediated endocytosis, passage through an acidic compartment, and delivery of Fragment A

10 associated ADP-ribosyltransferase to the cytosol of target cells, Bacha et al. (1988) supra.

#### Summary of the Invention

In general, the invention features a chimeric toxin including protein fragments joined together by  
15 peptide bonds. The chimeric toxin includes, in sequential order, beginning at the amino terminal end of the chimeric toxin:

(a) the enzymatically active Fragment A of diphtheria toxin;

20 (b) a first fragment including the cleavage domain 1<sub>1</sub> adjacent Fragment A of diphtheria toxin;

(c) a second fragment including at least a portion of the hydrophobic transmembrane region of Fragment B of diphtheria toxin, the second fragment also  
25 having a deletion, C-terminal to the transmembrane region, of at least 50, or more preferably of at least 80, diphtheria toxin amino acid residues, and the second fragment not including domain 1<sub>2</sub>; and

(d) a third fragment including a portion of a  
30 cell-specific polypeptide ligand e.g., an interleukin (preferably interleukin 2, or, epidermal growth factor (EGF), including at least a portion of the binding domain of the polypeptide ligand, that portion being effective to cause the chimeric toxin to bind

selectively to a predetermined class of cells to be attacked by enzymatically active Fragment A.

In preferred embodiments the chimeric toxin possesses at least one of, and more preferably at least two of, and even more preferably at least three of: greater toxicity to receptor-bearing cells than that of an analagous DAB<sub>486</sub>-containing-toxin (an analagous DAB<sub>486</sub>-containing toxin is a toxin which is identical to the chimeric toxin of the preferred embodiment except that DAB<sub>486</sub> replaces the fragments of DT recited in (a), (b), and (c) above, i.e., a toxin consisting of DAB<sub>486</sub> fused to the fragment defined in (d) above); a lower  $K_d$  (i.e., a greater binding affinity) for the receptor (i.e., the sites to which the third fragment (described above) binds on the cells to be attacked) than that of an analagous DAB<sub>486</sub>-containing-toxin; greater resistance to proteolytic degradation than that of DAB<sub>486</sub>-containing-toxin; greater resistance to the inhibition of its cytotoxicity by competitive inhibitors, e.g., the polypeptide of (d) above, than that exhibited by an analagous DAB<sub>486</sub>-containing-toxin; the ability to inhibit protein synthesis in target cells to a given degree by a period of exposure that is shorter than the period of exposure required by an analogous DAB<sub>486</sub>-containing-toxin to inhibit protein synthesis to the same degree; or the ability to effect a more rapid onset of the inhibition of protein synthesis than that seen in an analagous DAB<sub>486</sub>-containing-toxin.

Other preferred embodiments include: chimeric toxins wherein the fragment of Fragment B of diphtheria toxin does not include any diphtheria toxin sequences between the hydrophobic transmembrane region and amino acid residues 484 or 485 of native diphtheria toxin;

chimeric toxins lacking diphtheria toxin sequences C-terminal to amino acid residue 386 of native diphtheria toxin; and chimeric toxins including DAB<sub>389</sub> fused to the third fragment defined above.

5 Other preferred embodiments include: a chimeric toxin in which the portion of the polypeptide ligand is a portion of interleukin-2 effective to cause the chimeric toxin to bind to IL-2 receptor bearing cells, in particular, T cells; a chimeric toxin in which  
10 the portion of the polypeptide ligand is a portion of EGF effective to cause the chimeric toxin to bind to cells bearing the EGF receptor; the chimeric toxin DAB<sub>389</sub>-IL-2; and the chimeric toxin DAB<sub>389</sub>-EGF.

In other preferred embodiments in which the  
15 ligand is IL-2 or a portion thereof, the chimeric toxin possesses at least one of: greater toxicity to IL-2 receptor-bearing cells than that exhibited by DAB<sub>486</sub>-IL-2, a lower  $K_d$  for the IL-2 high affinity receptor than that of DAB<sub>486</sub>-IL-2, or a greater  
20 resistance to proteolytic degradation than that exhibited by DAB<sub>486</sub>-IL-2.

In other preferred embodiments in which the ligand is EGF or a portion thereof, the chimeric toxin possesses at least one of: greater toxicity to  
25 EGF-receptor-bearing cells than that exhibited by DAB<sub>486</sub>EGF; a lower  $K_d$  for the EGF receptor than that of DAB<sub>486</sub>EGF, greater resistance to the inhibition of its cytotoxicity by competitive inhibitors, e.g., EGF, than that of DAB<sub>486</sub>-EGF; the ability to inhibit  
30 protein synthesis in EGF receptor bearing cells to a given degree by a period of exposure that is shorter than the period of exposure required by DAB<sub>486</sub>EGF to inhibit protein synthesis to the same degree; or the ability to effect a more rapid onset of the inhibition

of protein synthesis in EGF-receptor-bearing cells than that seen in DAB<sub>486</sub>EGF.

The chimeric toxins of the invention are preferably encoded by fused genes which include regions encoding the protein fragments of the chimeric toxin,  
5 DNA sequences encoding the chimeric toxins of the invention, expression vectors encoding those DNA sequences, cells transformed with those expression vectors, and methods of producing the chimeric toxins  
10 including culturing cells transformed with expression vectors containing DNA encoding the chimeric toxins and isolating the chimeric toxins from the cells or their supernatants.

Native diphtheria toxin, as used herein, means  
15 the 535 amino acid diphtheria toxin protein secreted by Corynebacterium diphtheriae. The sequence of an allele of the gene which encodes native diphtheria toxin can be found in Greenfield et al. (1983) Proc. Natl. Acad. Sci. USA 80:6853-6857, hereby incorporated by reference.

Enzymatically active Fragment A, as used herein, means  
20 amino acid residues Gly 1 through Arg 193 of native DT, or an enzymatically active derivative or analog of the natural sequence. Cleavage domain 1<sub>1</sub>, as used herein, means the protease sensitive domain within the region  
25 spanning Cys 186 and Cys 201 of native DT. Fragment B, as used herein, means the region from Ser 194 through Ser 535 of native DT. The hydrophobic transmembrane region of Fragment B, as used herein, means the amino acid sequence bearing a structural similarity to the  
30 bilayer-spanning helices of integral membrane proteins and located approximately at or derived from amino acid residue 346 through amino acid residue 371 of native diphtheria toxin. Domain 1<sub>2</sub>, as used herein, means the region spanning Cys 461 and Cys 471 of native DT.

The generalized eukaryotic binding site of Fragment B, as used herein, means a region within the C-terminal 50 amino acid residues of native DT responsible for binding DT to its native receptor on the surface of eukaryotic cells. The chimeric toxins of the inventions do not include the generalized eukaryotic binding site of Fragment B.

Toxic or cytotoxic, as used herein, means capable of inhibiting protein synthesis in a cell, inhibiting cell growth or division, or killing a cell.

DAB<sub>486</sub> consists of, in the following order, methionine, and amino acid residues 1-485 of native DT.

DAB<sub>389</sub> consists of, in the following order, methionine, amino acid residues 1- 386 of native DT, and amino acid residues 484 - 485 of native DT.

DAB<sub>486</sub>-IL-2 is a fusion protein consisting of, in the following order, methionine, amino acid residues 1-485 of native DT, and amino acid residues 2-133 of IL-2. DAB<sub>485</sub>-IL-2 is identical except that it lacks the initial methionine residue.

DAB<sub>389</sub>-IL-2 consists of DAB<sub>389</sub> fused to amino acid residues 2-133 of IL-2.

DAB<sub>389</sub>EGF consists of DAB<sub>389</sub> fused to EGF.

Receptor means the site to which the cell-specific polypeptide ligand (described in (d) above) binds.

Chimeric toxins of the invention display one or more of the following advantages: greater toxicity than that of an analagous DAB<sub>486</sub>-containing toxin; a greater affinity for the receptor than that of an analagous DAB<sub>486</sub>-containing toxin; when expressed in the cytoplasm of E.coli, greater resistance to proteolytic degradation than that exhibited by an analagous DAB<sub>486</sub>-containing toxin; greater resistance



to the inhibition of its cytotoxicity by competitive inhibitors, e.g., the polypeptide of (d) above, than that exhibited by an analogous DAB<sub>486</sub>-containing toxin; the ability to inhibit protein synthesis in target cells to a given degree by a period of exposure that is shorter than the period of exposure required by an analogous DAB<sub>486</sub>-containing-toxin to inhibit protein synthesis to the same degree; or the ability to effect a more rapid onset of the inhibition of protein synthesis than that seen in an analogous DAB<sub>486</sub>-containing-toxin.

Aberrant expression of the epidermal growth factor receptor is a characteristic of several malignancies including those of the breast, bladder, prostate, lung and neuroglia. Chimeric toxins of the invention allow therapeutic targeting the cytotoxic action of diptheria toxin to EGF receptor positive tumor cells. In these chimeric toxins the sequences for the binding domain of diptheria toxin have been replaced by those for human EGF. These chimeric toxins inhibit protein synthesis by the same mechanism as diptheria toxin and are specifically cytotoxic for human tumor cells which express elevated levels of EGF receptors. The uptake of these chimeric toxins occur with kinetics which permit use of this molecule as a powerful therapeutic agent for treatment of malignancies characterized by EGF receptor expression.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments and from the claims.

#### Description of the Preferred Embodiments

The drawings will first be briefly described.

Drawings

Fig. 1 is a diagram of the DT molecule and of various fusion proteins.

Fig. 2 is a depiction of the construction of the plasmids of a preferred embodiment.

Fig. 3 is a restriction map of DNA sequences encoding various chimeric toxins.

Fig. 4 is a graph of the effects of varying doses of chimeric toxins on cultured cells.

Fig. 5 is a graph of the ability of chimeric toxins to competitively displace [ $^{125}$ I]-labeled IL-2 from the high affinity IL-2 receptor.

Fig. 6 is the sequence of a synthetic EGF gene.

Fig. 7 is a diagrammatic representation of DAB<sub>486</sub>EGF and DAB<sub>389</sub>EGF.

Fig. 8 is a graph showing the effect of EGF on DAB<sub>486</sub>EGF cytotoxicity.

Fig. 9 is a graph showing the effect of EGF on DAB<sub>389</sub>EGF cytotoxicity.

Fig. 10 is a graph showing the effect of EGF and DAB<sub>389</sub>EGF on the EGF binding capacity of A431 cells.

Fig. 11 is a graph showing the ability of EGF or DAB<sub>389</sub>EGF to displace [ $^{125}$ I] EGF from EGF receptors.

Fig. 12 is a graph of the effect of length of exposure to DAB<sub>486</sub>EGF on the inhibition of protein synthesis.

Fig. 13 is a graph of the effect of length of exposure to DAB<sub>389</sub>EGF on the inhibition of protein synthesis.

Fig. 14 is a graph of the kinetics of the inhibition of protein synthesis on cells incubated with DAB<sub>486</sub>EGF or DAB<sub>389</sub>EGF.

Structure and Synthesis of chimeric toxin DAB<sub>486</sub>-IL-2

DAB<sub>486</sub>-IL-2 is a chimeric toxin consisting of Met followed by amino acid residues 1 through 485 of mature DT fused to amino acid residues 2 through 133 of IL-2. The DT portion of the chimeric toxin DAB<sub>486</sub>-IL-2 includes all of DT fragment A and the portion of DT fragment B extending to residue 485 of mature native DT. Thus DAB<sub>486</sub>-IL-2 extends past the disulfide bridge linking Cys 461 with Cys 471. See Fig. 1a for the structure of DT. (The nomenclature adopted for IL-2-toxin is DAB<sub>486</sub>-IL-2, where D indicates diphtheria toxin, A and B indicate wild type sequences for these fragments, and IL-2 indicates human interleukin-2 sequences. Mutant alleles are indicated by a number in parentheses following DAB. The numerical subscript indicates the number of DT-related amino acids in the fusion protein. Since the deletion of the tox signal sequence and expression from the trc promoter results in the addition of a methionine residue to the N-terminus, the numbering of DAB-IL-2 fusion toxins is +1 out of phase with that of native diphtheria toxin.)

pDW24, which carries DAB<sub>486</sub>-IL-2 was constructed as follows. pUC18 (New England BioLabs) was digested with PstI and BglI and the PstI-BglI fragment carrying the E.coli origin of replication, the polylinker region, and the 3' portion of the  $\beta$ -lactamase gene ( $\text{amp}^r$ ) was recovered. Plasmid pKK-233-2 (Pharmacia) was digested with PstI and BglI and the PstI-BglI fragment carrying, two transcription terminators and the 5' portion of the  $\beta$ -lactamase gene was recovered. pDW22 was constructed by ligating these two recovered fragments together.

pDW23 was constructed by isolating a BamHI-SalI fragment encoding human IL-2 from plasmid pDW15

(Williams et al. (1988) Nucleic Acids Res. 16:10453-10467) and ligating it to BamHI/SalI digested pDW22 (described above).

pDW24 was constructed as follows. A BamHI-NcoI  
5 fragment carrying the trc promoter and translational  
initiation codon (ATG) was isolated from plasmid  
pKK233-2 (Pharmacia). The DNA sequence encoding amino  
acid residues 1 through 485 of DT was obtained by  
digesting pABC508 (Williams et al. (1987) Protein  
10 Engineering 1:493-498) with SphI and HaeII and  
recovering the HaeII-SphI fragment containing the  
sequence encoding amino acid residues 1 through 485 of  
DT. A NcoI/HaeII linker (5'CCATGGGCGC 3') was ligated  
to the HaeII-SphI fragment and that construction was then  
15 ligated to the previously isolated BamHI-NcoI fragment  
carrying the trc promoter. This results in a Bam  
HI-SphI fragment bearing, in the following order, the  
trc promoter, the NcoI site (which supplies the ATG  
initiator codon for Met), and the sequence encoding  
20 residues 1 through 485 of native DT. This fragment was  
inserted into pDW23 that had been digested with Bam HI  
and SphI. The resulting plasmid was designated pDW24.  
The fusion protein (DAB<sub>486</sub>-IL-2) encoded by pDW24 is  
expressed from the trc promoter and consists of Met  
25 followed by amino acids 1 through 485 of mature DT fused  
to amino acids 2 through 133 of human IL-2.

The sequence of DT is given in Greenfield et  
al. (1983) supra. The sequence encoding IL-2 was  
synthesized on an Applied Biosystems DNA-Synthesizer, as  
30 described in Williams et al. (1988) Nucleic Acids Res.  
16:10453-10467, hereby incorporated by reference. The  
sequence of IL-2 is found in Williams et al. (1988)  
Nucleic Acids Res. 16:10453-10467. Fusion of the  
sequence encoding mature DT to ATG using an

oligonucleotide linker is described in Bishai et al. (1987) J. Bact. 169:5140-5151, hereby incorporated by reference.

pDW24 is shown in Fig. 2. The insert corresponding to DAB<sub>486</sub>-IL-2 is shown as a heavy line. In Fig. 2 filled circles indicate NcoI sites, open circles indicate NsiI sites, open diamonds indicate ClaI sites, filled squares indicate HpaII sites, open squares indicate SphI sites, and filled triangles indicate SalI sites.

Oligonucleotides and nucleic acids were manipulated as follows. Oligonucleotides were synthesized using cyanoethyl phosphoramidite chemistry on an Applied Biosystems 380A DNA synthesizer (Applied Biosystems Inc., Foster City, CA). Following synthesis, oligonucleotides were purified by chromatography on Oligonucleotide Purification Cartridges (Applied Biosystems Inc., Foster City, CA) as directed by the manufacturer. Purified oligonucleotides were resuspended in TE buffer (10 mM Tris base, 1 mM EDTA, pH 8.0). To anneal complementary strands, equimolar concentrations of each strand were mixed in the presence of 100 mM NaCl, heated to 90°C for 10 min, and allowed to cool slowly to room temperature.

Plasmid DNA was purified by the alkaline lysis/cesium chloride gradient method of Ausebel et al. (1989) Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. DNA was digested with restriction endonucleases as recommended by the manufacturer (New England Biolabs, Beverly, MA and Bethesda Research Laboratories, Gaithersburg, MD). Restriction fragments for plasmid construction were extracted from agarose-TBE gels, ligated together (with or without oligonucleotide linkers) and used to transform E. coli using standard

methods. Ausebel et al (1989) supra and Maniatis et al. (1982), Molecular Cloning Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. Plasmid DNA sequencing was performed according to the dideoxy chain termination method of Sanger et al. (1987) Proc. Nat'l Acad. Sci USA 74:5463-5467, as modified by Kraft et al. (1988) Bio Techniques 6:544-547, using Sequenase (United States Biochemicals, Cleveland, OH).

#### Structure of Improved Diphtheria-IL-2 Chimeric Toxins

Expression and purification of chimeric toxins was as follows. All DT-related IL-2 fusion proteins used herein were expressed in the cytoplasm of E. coli strain JM101 from the trc promoter, Amann et al. (1985), Gene 40:183-190, hereby incorporated by reference. Recombinant E. coli were grown in M9 minimal medium (Maniatis et al. (1982) supra) supplemented with 10 mg/ml casamino acids (Difco, Detroit, MI), 50 µg/ml ampicillin, and 0.5 ng/ml thymine in 10 liter volumes in a Microgen Fermentor (New Brunswick Scientific, Edison, N.J.). Bacterial cultures were grown at 30°C, and sparged with air at 5 L/min. When the absorbance ( $A_{590\text{nm}}$ ) of the culture reached 0.3, expression of chimeric tox gene was induced by the addition of isopropyl-β-D- thiogalactopyranoside. Two hours after induction, bacteria were harvested by centrifugation, resuspended in buffer #101 (50 mM  $\text{KH}_2\text{PO}_4$ , 10 mM EDTA, 750 mM NaCl, 0.1% Tween 20, pH 8.0), and lysed by sonication (Branson Sonifier). Whole cells and debris were removed by centrifugation at 27,000 x g, and the clarified extract was then filter sterilized and applied to an anti-diphtheria toxin immunoaffinity column. Bound proteins were eluted with 4M guanidine hydrochloride, reduced by the addition of β-mercaptoethanol to 1% and then sized by high pressure

liquid chromatography on a 7.5 x 600 mm G4000PW column (TosoHass). Prior to use, fusion toxins were exhaustively dialysed against HEPES buffered Hank's balanced salt solution (Gibco), pH 7.4. Purified diphtheria toxin was purchased from List Biological Laboratories (Campbell, CA). For the production of the non-toxic CRM1001, C7(Btox-1001) was grown in 100 ml volumes of C-Y medium (Rappuoli et al. (1983) J. Bact. 153:1201-1210) in 2-liter Erlenmeyer flasks at 35°C for 20 hrs with shaking (240 rpm). Bacteria were removed by centrifugation at 20,000 x g for 15 min. CRM1001 was precipitated from the culture medium by the addition of  $\text{NH}_4\text{SO}_4$  to 70% saturation, and collected by centrifugation. Following dialysis against 10 mM phosphate buffer, pH 7.2, CRM1001 was purified by ion exchange chromatography on DE-52 cellulose as previously described by Pappenheimer et al. (1972), Immunochem. 9:891-906. The concentration of all purified proteins was determined by using Pierce Protein Assay reagent (Pierce Chemical Co., Rockford, IL).

DAB(1001)<sub>486</sub>-IL-2 is a chimeric toxin identical to DAB<sub>486</sub>-IL-2 except for the disruption of the disulfide bridge between Cys462 and Cys472 in DAB(1001)<sub>486</sub>-IL-2. DAB(1001)<sub>486</sub>-IL-2 was constructed by replacing the 587 basepair (bp) ClaI-SphI restriction fragment which encodes most of fragment B of DT) of plasmid pDW24 (which carries DAB<sub>486</sub>-IL-2) with the analogous fragment from DNA encoding the TOX-1001 mutant allele of DT. TOX-1001 encodes non-toxic diphtheria toxin-related protein CRM1001 and has been shown to result from a single point mutation which changes Cys471 to Tyr471, Rappuoli et al. (1986) In Protein Carbohydrate Interactions in Biological Systems, Academic Press, Inc., London, pp. 295-296, hereby

incorporated by reference. Fig. 3 depicts the restriction maps of DNA encoding DAB<sub>486</sub>-IL-2 and the corresponding fusion protein encoded by DAB<sub>486</sub>-IL-2. (In Fig. 3 stippled boxes between the NsiI and HpaII restriction endonuclease sites designate the diphtheria toxin fragment B-related sequences which encode the membrane associating domains. The amphipathic domain is encoded between the NsiI and ClaI sites, and the putative membrane spanning domains are encoded between the ClaI and HpaII sites. Hatched boxes indicate the relative position of internal in-frame deletion mutations.) The construction of pDW26, which encodes the chimeric toxin with the Cys 472 to Tyr 472 mutation, is shown in Fig. 2. Following ligation and transformation, the DNA sequence of the tox-1001 portion of the gene fusion DAB (1001)<sub>486</sub>-IL-2 was determined in order to insure that the Cys471 to Tyr471 mutation was recloned. E. coli (pDW26), was grown in M9 minimal media, cells were harvested, lysed and the fusion toxin, designated DAB(1001)<sub>486</sub>-IL-2, was purified by immunoaffinity chromatography and HPLC.

The dose response capacity of DAB<sub>486</sub>-IL-2, CRM1001, and DAB(1001)<sub>486</sub>-IL-2 to block [<sup>14</sup>C]-leucine incorporation by high affinity IL-2 receptor bearing HUT 102/6TG cells was determined. As anticipated, DAB<sub>486</sub>-IL-2 was highly toxic for these cells (IC<sub>50</sub> = 4 x 10<sup>-10</sup>M); whereas, CRM1001 was found to be non-toxic. In marked contrast to CRM1001, however, the fusion toxin which carries the Cys472 to Tyr472 mutation, DAB(1001)<sub>486</sub>-IL-2, was found to be as toxic for HUT 102/6TG cells as the wild type DAB<sub>486</sub>-IL-2. These results demonstrate that the fragment B disulfide bond is not required for biological activity of the fusion toxin.



HUT 102/6TG cytotoxicity assays were performed as follows. Cultured HUT 102/6TG cells were maintained in RPMI 1640 medium (Gibco, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Cellec, GIBCO), 2 mM glutamine, and penicillin and streptomycin to 50 IU and 50 µg/ml, respectively. For cytotoxicity assays, cells were seeded in 96-well V-bottomed plates (Linbro-Flow Laboratories, McLean, VA) at a concentration of  $5 \times 10^4$  per well in complete medium. Toxins, or toxin-related materials, were added to varying concentrations ( $10^{-12}$ M to  $10^{-6}$ M) and the cultures were incubated for 18 hrs at 37°C in a 5% CO<sub>2</sub> atmosphere. Following incubation, the plates were centrifuged for 5 min. at 170 x g and the medium removed and replaced with 200 µl leucine-free medium (MEM, Gibco) containing 1.0 µCi/ml [<sup>14</sup>C]-leucine (New England Nuclear, Boston, MA). After an additional 90 min. at 37°C, the plates were centrifuged for 5 min. at 170 x g, the medium was removed and the cells were lysed by the addition of 4 M KOH. Protein was precipitated by the addition of 10% trichloroacetic acid and the insoluble material was then collected on glass fiber filters using a cell harvester (Skatron, Sterling, VA). Filters were washed, dried, and counted according to standard methods. Cells cultured with medium alone served as the control. All assays were performed in quadruplicate.

Since the disulfide bond between Cys462 - Cys472 was not required for the cytotoxic action of DAB<sub>486</sub>-IL-2, it was of interest to determine what DT fragment B sequences were essential for the delivery of fragment A to the cytosol. Several in-frame deletion mutations were introduced into the fragment B encoding portion of the DAB<sub>486</sub>-IL-2 toxin gene, Figs. 1b, 2,

and 3. Fig. 1b shows the structure of DAB<sub>486</sub>-IL-2 and various mutants derived from DAB<sub>486</sub>-IL-2. In Fig. 1b a wide bar indicates the fusion protein, narrow connecting lines represent deletions, numbers above the bars are amino acid residue numbers in the DAB nomenclature, numbers below the bars correspond to the amino acid residue numbering of native DT, cross hatching indicated amphipathic regions, darkened areas correspond to the transmembrane region, IL-2-2-133 indicates amino acid residues 2-133 of IL-2, Ala = alanine, Asn = asparagine, Asp = aspartic acid, Cys = cysteine, Gly = glycine, His = histidine, Ile = isoleucine, Met = methionine, Thr = threonine, Tyr = tyrosine, and Val = valine.

The first mutant, DAB<sub>389</sub>-IL-2 was constructed by removing a 309 bp HpaII - SphI restriction fragment from pDW24 and replacing it with oligonucleotide linker 261/274 (Table 1) to generate plasmid pDW27 (Fig. 1). This linker restores fragment B sequences from Pro383 to Thr387, and allows for in-frame fusion to IL-2 sequences at this position. Thus, in DAB<sub>389</sub>-IL-2 the 97 amino acids between Thr387 and His485 have been deleted.

Table 1. Oligonucleotide linkers.

construct	oligonucleotide identification number	linker
5 DAB <sub>389</sub> -IL-2	274 261	5'-CG GGT CAC AAA ACG CAT G-3' CCA GTG TTT TGC 1/2 <u>HpaII</u> 1/2 <u>SphI</u>
DAB <sub>295</sub> -IL-2	292 293	5'-C GAT GGT GTG CAT G-3' TA CCA CAC 1/2 <u>ClaI</u> 1/2 <u>SphI</u>
10 DAB( $\Delta$ 205- 289) <sub>486</sub> -IL-2	337 338	5'-TA AAT AT-3' ACG TAT TTA TAG C 1/2 <u>NsiI</u> 1/2 <u>ClaI</u>
DAB( $\Delta$ 205- 289) <sub>389</sub> -IL2	337 338	" "

15 In a similar fashion, a 191 amino acid in-frame deletion was constructed by removing a ClaI - SphI restriction fragment from pDW24 and replacing it with the 292/293 oligonucleotide linker (Table 1) to form  
20 plasmid pDW28 which encodes DAB<sub>295</sub>-IL-2. In this case, the in-frame deletion encompasses the putative membrane-spanning helices that have been predicted by Lambotte et al. (1980) J. Cell. Biol. 87:837-840, to  
25 play a role in the delivery of fragment A to the eukaryotic cell cytosol.

Purified, DAB<sub>389</sub>-IL-2 and DAB<sub>295</sub>-IL-2 were found to have electrophoretic mobilities of 57 kDa and 47 kDa, respectively. The dose response analysis on HUT  
30 102/6TG cells is shown in Fig. 4. In Fig. 4 DAB<sub>486</sub>-IL-2 is indicated by filled squares; DAB<sub>389</sub>-IL-2 is indicated by filled circles; DAB<sub>295</sub>-IL2 is indicated by open circles; DAB( $\Delta$ 205-289)<sub>486</sub>-IL-2 (see below)

is indicated by open squares; and DAB( $\Delta$ 205-289)<sub>389</sub>-IL-2 (see below) is indicated by open triangles. DAB<sub>486</sub>-IL-2 and DAB<sub>389</sub>-IL-2 exhibited an IC<sub>50</sub> of approximately  $4 \times 10^{-10}$ M and  $1 \times 10^{-10}$ M, respectively. In marked contrast, the IC<sub>50</sub> of DAB<sub>295</sub>-IL-2 was approximately 1,000-fold lower ( $4 \times 10^{-7}$ M). These results suggest that fragment B sequences between Thr 387 and His 486 do not play a major role in the delivery of fragment A to the cytosol. Sequences between Ser292 and Thr387 on the other hand are essential for the efficient delivery of fragment A.

Surprisingly, DAB<sub>389</sub>-IL-2 possessed much greater activity than did DAB<sub>486</sub>-IL-2. DAB<sub>389</sub>-IL-2, which lacks native DT residues 387 through 483, and which has increased toxic activity, leaves the hydrophobic transmembrane segment located approximately between native DT residues 346 and 371 intact. See Lambotte et al. (1980) J. Cell Biol. 87:837-840, hereby incorporated by reference, for a characterization of the transmembrane region. DAB<sub>295</sub>-IL-2, which removes native DT residues 291 through 481, and which has greatly reduced toxicity, removes the transmembrane region (346-371).

In order to rule out the possibility that the reason for the low potency of DAB<sub>295</sub>-IL-2 for HUT 102/6TG cells was related to altered binding to the high affinity IL-2 receptor, we have conducted a series of competitive displacement experiments using [<sup>125</sup>I]-rIL-2. Fig. 5 shows the competitive displacement of [<sup>125</sup>I]-labeled IL-2 from the high affinity IL-2 receptor by unlabeled rIL-2 depicted by filled circles; DAB<sub>486</sub>-IL-2 depicted by open triangles; DAB<sub>389</sub>-IL-2 depicted by closed squares;

DAB<sub>295</sub>-IL-2 depicted by closed triangles;  
DAB( $\Delta$ 205-289)<sub>486</sub>-IL-2 (see below) depicted by open  
circles; and DAB( $\Delta$ 205-289)<sub>389</sub>-IL-2 (see below)  
depicted by open squares. The concentration of  
5 [ <sup>125</sup>I ]-IL-2 used was 10 pM and the specific activity  
was approximately 0.7  $\mu$ Ci/pmol. As shown in Table 2,  
both DAB<sub>389</sub>-IL-2 and DAB<sub>295</sub>-IL-2 were found to have  
an apparent  $K_d$  that is approximately 3-times lower  
than that of DAB<sub>486</sub>-IL-2 ( $K_d = 8 \times 10^{-9}$  M vs.  $K_d$   
10  $= 2.5 \times 10^{-8}$  M). It is particularly significant that  
competitive displacement experiments showed that both  
DAB<sub>389</sub>-IL-2 and DAB<sub>295</sub>-IL-2 bind more avidly to the  
high affinity IL-2 receptor than does DAB<sub>486</sub>-IL-2 ( $K_d =$   
8  $\times 10^{-9}$  and 8.4  $\times 10^{-9}$  M vs.  $K_d = 2.5 \times 10^{-8}$  M).  
15 These results provide evidence that fusion of IL-2  
sequences to toxophores of smaller mass may serve to  
position the IL-2 binding domain for more favorable  
receptor interaction.

It is of interest to note that while  
20 DAB<sub>295</sub>-IL-2 binds more avidly to the high affinity  
IL-2 receptor than DAB<sub>486</sub>-IL-2, its cytotoxic activity  
is at least 1,000-fold lower (Fig. 4). These results  
indicated that avid binding to the target receptor is  
not in itself sufficient for the biologic activity of  
25 the DT-related IL-2 fusion toxins, and that fragment B  
sequences between Ser292 and Thr387 are essential for a  
post-receptor binding event in the intoxication process.

Table 2. Relative ability of rIL-2 and DAB-IL-2 related fusion proteins to displace [ $^{125}$ I]--rIL-2 from high affinity IL-2 receptors on HUT 102/6TG cells

5	unlabeled ligand	apparent $K_d$	$K_d$ DAB-IL-2/rIL-2
	rIL-2	$1.7 \times 10^{-10}$	-
	DAB <sub>486</sub> -IL-2	$2.5 \times 10^{-8}$	147
	DAB <sub>389</sub> -IL-2	$8.0 \times 10^{-9}$	47
10	DAB <sub>295</sub> -IL-2	$8.4 \times 10^{-9}$	49
	DAB( $\Delta$ 205-289) <sub>486</sub> -IL-2	$1.0 \times 10^{-7}$	588
	DAB( $\Delta$ 205-289) <sub>389</sub> -IL-2	$2.9 \times 10^{-8}$	170

15 Competitive displacement of [ $^{125}$ I]-rIL-2 by  
 rIL-2 and DAB-IL-2 fusion toxins was determined as  
 follows. The radiolabeled IL-2 binding assay was  
 performed essentially as described by Wang et al. (1987)  
 J. Exp. Med. 166:1055-1069. Cells were harvested and  
 20 washed with cell culture medium. HUT 102/6TG cells were  
 resuspended to  $5 \times 10^6$  per ml and incubated with  
 [ $^{125}$ I]-rIL-2 (0.7  $\mu$ Ci/pmol) in the presence or  
 absence of increasing concentrations of unlabeled rIL-2  
 or the DAB-IL-2 fusion toxins for 30 min. at 37°C under  
 25 5% CO<sub>2</sub>. The reaction was then overlayed on a mixture  
 of 80% 550 fluid (Accumetric Inc., Elizabethtown, KN) :  
 20% parafin oil (d = 1.03 g/ml) and microcentrifuged.  
 The aqueous phase and the pellet of each sample,  
 representing free and bound ligand, respectively, was  
 30 then counted in a Nuclear Chicago gamma counter.  
 Apparent dissociation constants,  $K_d$ , were determined

from the concentrations of unlabeled ligand required to displace 50% of radiolabeled rIL-2 binding to receptors.

In order to test the hypothesis that an amphipathic region (amino acids 210-252 in DAB<sub>486</sub>-IL-2) plays a role in the intoxication process, in-frame deletions of the 85 amino acid encoding region from NsiI to ClaI of both pDW24 and pDW27 to form pDW30 (containing DAB( $\Delta$ 205-289)<sub>486</sub>-IL-2) and pDW31 (containing DAB( $\Delta$ 205-289)<sub>389</sub>-IL-2), respectively (Figs. 2 and 3; Table 1) were constructed. Following ligation and transformation, the DAB-IL-2 related fusion proteins were expressed and purified, as described above. As shown in Figure 4, the deletion of fragment B sequences which include the amphipathic region result in a marked loss of cytotoxic activity against high affinity IL-2 receptor positive cells in vitro. It is of interest to note that DAB ( $\Delta$ 205-289)<sub>389</sub>-IL-2 was found to displace radiolabeled IL-2 from the high affinity receptor almost as well as DAB<sub>486</sub>-IL-2; whereas, DAB( $\Delta$ 205-289)<sub>486</sub>-IL-2 was found to bind 4-fold less avidly to the receptor (Fig. 5).

#### Increased Resistance to Proteolytic Degradation

The chimeric toxin encoded by DAB<sub>389</sub>-IL-2 is more resistant to proteolytic degradation than is the chimeric toxin encoded by DAB<sub>486</sub>-IL-2. When purified, as described above, and analysed on SDS-polyacrylamide gels, the DAB<sub>389</sub>-IL-2 hybrid toxin is accompanied by very few degradation products (as evidenced by the relative absence of bands of smaller size than that of the intact chimeric toxin). Purified DAB<sub>486</sub>-IL-2 on the other hand is accompanied by numerous dark bands of lower molecular weight than the intact chimeric toxin. These lower molecular weight bands react with

anti-DAB<sub>486</sub>-IL-2 antibodies, supporting the conclusion that they are degradation products.

Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemmli (1970) Nature 227:680-685 using 12% gels and a Mini-Protein II gel apparatus (BioRad). Proteins were fixed in 12.5% trichloroacetic acid for 5 min and stained with Coomassie brilliant blue according to the Diezal procedure, Diezal et al. (1972) Anal. Biochem. 48:617-624.

#### Construction of Fusion Genes Encoding DT-EGF Chimeric Toxins

DAB<sub>486</sub> EGF and DAB<sub>389</sub> EGF can be constructed in a manner analogous to that in which DAB<sub>486</sub>-IL-2 and DAB<sub>389</sub>-IL-2 were constructed, by methods known to those skilled in the art. To construct a plasmid encoding DAB<sub>486</sub> fused to EGF, plasmid pDW24 (which encodes DAB<sub>486</sub> fused to IL-2) is digested with SphI and HindIII to remove the IL-2 coding sequence. The resulting pDW24 SphI-HindIII fragment containing the sequence encoding DT residues 1-485 is ligated to a synthetic SphI-HindIII fragment encoding EGF to yield a plasmid encoding DAB<sub>486</sub> fused to EGF. The EGF fragment, shown in Fig. 6, was synthesized, as described, using preferred codons for expression in E.coli (see Grosjean et al. (1982) Gene 18:199-209, hereby incorporated by reference). The synthetic fragment includes appropriate linkers at the 5' and 3' ends for insertion into the plasmid and for in-frame fusion to the DT coding sequence.

To construct a plasmid encoding DAB<sub>389</sub> fused to EGF a similar protocol can be followed, except that pDW27 (which encodes DAB<sub>389</sub> fused to IL-2) is used in place of pDW24. The IL-2 encoding DNA is removed from



pDW27 by digestion with SphI and HindIII and EGF encoding DNA is inserted in its place, resulting in a DAB<sub>389</sub> fused in frame to EGF. The same synthetic EGF fragment used in the construction of the DAB<sub>489</sub>EGF fusion (Fig. 6) can be used.

Those skilled in the art will realize that the protocols given above are not the only way of making the chimeric toxins of the invention. Refinements include changes in pDW24, pDW27, and plasmids derived therefrom directed toward compliance with the Good Manufacturing Practises of the Food and Drug Administration, e.g., the inclusion of the lacI<sup>q</sup> gene (Amersham) and the replacement of the ampicillin resistance gene (amp<sup>r</sup>) with the gene for neomycin/kanamycin resistance from Tn5 (Pharmacia) in the plasmids that are used for expression of the chimeric toxins of the invention. These alterations can be performed without undue experimentation by one skilled in the art.

#### The Biological Activity of DT-EGF Chimeric Toxins

DAB<sub>486</sub>EGF and DAB<sub>389</sub>EGF are the products of fusion genes in which the receptor binding domain of DT has been removed and replaced with DNA encoding human EGF. As shown in Fig. 7, the resulting proteins contain the enzymatically active fragment A of DT and the lipid associating domains of fragment B of DT required for translocation of fragment A into the cytosol.

DAB<sub>389</sub>EGF differs from DAB<sub>486</sub>EGF by the deletion of the 97 amino acids immediately 5' to amino acid residue 484 of DT. The EGF portion of both DAB<sub>486</sub>EGF and DAB<sub>389</sub>EGF governs receptor binding. Thus, these molecules have the potential to specifically target the cytotoxicity of DT to tumor cells characterized by EGF receptor expression.

DT-EGF Chimeric Toxins Are Toxic to  
EGF-Receptor-Bearing Cells. The cytotoxicity of  
DAB<sub>486</sub>EGF for a panel of human cell lines was assessed  
and compared to A431 cells (ATCC CRL 1555), a human  
5 epidermoid carcinoma cell line with a high number of EGF  
receptors. The results are shown in Table 3. Included  
in the study were human tumor cell lines which have been  
reported to express high numbers of EGF receptors (e.g.,  
BT-20, HeLa, LNCaP and U-87 MG) as well as human tumor  
10 cell lines (e.g., C91/PL, BeWo and A375) and normal  
tissue cell lines (e.g., WI-38, Hs 67 and HEPM)  
expressing few or no EGF receptors. Cytotoxicity was  
evaluated as follows. Cells were plated in triplicate  
wells of 96 well plates with DAB<sub>486</sub>EGF in assay medium  
15 appropriate to the cell type (see Table 3). DAB<sub>486</sub>EGF  
concentrations were between  $10^{-15}$  and  $10^{-7}$  M.  
Following a 20-hour incubation, cells were labeled with  
[<sup>14</sup>C]leucine, trypsinized, harvested onto glass fiber  
filter mats and counted to determine the percent of  
20 control incorporation. Cell lines exhibiting an IC<sub>50</sub>  
for DAB<sub>486</sub>EGF of less than 0.5 nM were considered  
sensitive.

25

30

Table 3: The effect of a DT-EGF chimeric toxin on various cell lines

Tumor Cell lines			
	<u>Cell Line</u>	<u>Tissue/Type</u>	<u>Sensitivity</u>
5	A431	vulval epidermoid carcinoma	+
	A549	lung carcinoma	+
	KB	oral epidermoid carcinoma	+
10	BT-20	breast adenocarcinoma	+
	HeLa S3	cervical carcinoma	+
	T47D	breast ductal carcinoma	+
	LNCaP.FG	prostate carcinoma	+
15	HOS	osteosarcoma	+
	U-87 MG	glioblastoma/astrocytoma	+
	C91/PL	HTLV-1 transformed T cell	-
20	BeWo	choriocarcinoma	-
	A375	malignant melanoma	-
	MCF-7	breast adenocarcinoma	-
	SNU-C2B	cecum carcinoma	-
25	Normal Cell Lines		
	<u>Cell Line</u>	<u>Tissue</u>	<u>Sensitivity</u>
	WI-38	diploid lung fibroblast	-
30	Hs 67	thymus	-
	CCD-18Co	colon fibroblast	-
	HISM	smooth muscle, jejunum	-
	FH74s Int	fetal small intestine	-
	HEPM	embryonic palatal mesenchyme	-

Growth conditions and passage schedules used were those defined by ATCC (except as noted below). Culture media were as follows: A431 (ATCC CRL1555), DMEM + 10% FCS; A549 (ATCC CCL185) Ham's F12 + 10% FCS; 5 KB (ATCC CCL17), DMEM + NEAA + 10% FCS; BT-20 (ATCC HTB19), MEM + 10% FCS; HeLa S3 (ATCC CCL2.2), Ham's F12 + 10% FCS; T47D (ATCC HTB133), RPMI 1640 + 10% FCS; LNCaP.FG (ATCC CRL1740), RPMI 1640 + 10% FCS; HOS (ATCC CRL1543), MEM + 10% FCS; U-87 MG (ATCC HTB14), MEM + 10% 10 FCS; C91/PL (from Robert Swartz, NEMC, Boston, MA, see Bacha et al. (1988) J. Exp. Med. 167:612 for growth conditions), RPMI 1640 + 15% FCS; BeWo (ATCC CCL98), Ham's F12 + 15% FCS; A375 (ATCC CRL 1619), DMEM + 10% FCS; MCF-7 (ATCC TB22) MEM + 10% FCS; SNU-C2B (ATCC 15 CCL250) RPMI 1640 + 10% FCS; WI-38 (ATCC CCL75), Eagle's Basal + 10% FCS; Hs 67 (ATCC HTB 163), DMEM + 10% FCS; CCD-18Co (ATCC CRL 1459), MEM + 10% FCS; HISM (ATCC CRL 1692), DMEM + 10% FCS; FHs74Int (ATCC CCL241), DMEM + 10% FCS; HEPM (ATCC CRL 1486), MEM + 10% FCS. 20 DMEM = Dulbecco's modified Eagles Medium; MEM = Minimum Essential Medium; NEAA = Non-Essential Amino Acids; FCS = Fetal Calf Serum; ATCC = American Type Culture Collection.

25 To demonstrate that the cytotoxic action of DAB<sub>486</sub>EGF and DAB<sub>389</sub>EGF are mediated selectively by the EGF receptor, A431 cells were plated in triplicate wells of 96 well plates with DAB<sub>486</sub>EGF (Fig. 8) or 30 DAB<sub>389</sub>EGF (Fig. 9) in the presence of the specific competitor of the EGF receptor, human EGF (Upstate Biotechnologies, Inc.) ( $10^{-7}$  M), in assay medium (DMEM + 10% FCS). In Fig. 8 solid squares indicate DAB<sub>486</sub>EGF and solid triangles indicate DAB<sub>486</sub>EGF + EGF. In

Fig. 9 solid squares indicate DAB<sub>389</sub>EGF and solid triangles indicate DAB<sub>389</sub>EGF + EGF. Following a 20-hour incubation at 37°C, cells were labeled with [<sup>14</sup>C]leucine, trypsinized, harvested onto glass fiber filter mats and counted to determine the percent of control incorporation. The results show that, in the absence of EGF, DAB<sub>486</sub>EGF and DAB<sub>389</sub>EGF inhibit protein synthesis with an IC<sub>50</sub> of  $3 \times 10^{-12}$  M and  $3 \times 10^{-13}$  M, respectively. EGF almost completely abolishes this activity. Likewise, anti-EGF (Biomedical Technologies, Inc.) and anti-EGF receptor (Upstate Biotechnologies, Inc.) also abolish the cytotoxicity of DAB<sub>486</sub>EGF and DAB<sub>389</sub>EGF while the nonspecific competitors, transferrin (Sigma) anti-transferrin (Dako), and anti-transferrin receptor (Dako), have no effect. These results demonstrate that DAB<sub>486</sub>EGF and DAB<sub>389</sub>EGF are potent and specific cytotoxic agents. Note that DAB<sub>389</sub>EGF is approximately 10 times more potent than DAB<sub>486</sub>EGF.

DAB<sub>389</sub>EGF, like EGF, induces down regulation of the EGF receptor, providing further evidence for the EGF receptor-specific nature of DT-EGF chimeric toxins. Binding and internalization of EGF induces down regulation of the EGF receptor which can be detected as a decrease in [<sup>125</sup>I]EGF binding capacity (Krupp et al. (1982) J. Biol. Chem. 257:11489). The ability of DAB<sub>389</sub>EGF to induce EGF receptor internalization and subsequent down regulation was evaluated and compared to that induced by native EGF. The results are shown in Fig. 10. In Fig. 10 open squares indicate EGF and closed diamonds indicate DAB<sub>389</sub>EGF. A431 cells in triplicate wells of 24 well plates were preincubated with EGF or DAB<sub>389</sub>EGF ( $10^{-8}$  M) for the indicated times in DMEM + 0.1% BSA (bovine serum albumin) at

37°C. The cells were then placed on ice and acid stripped (with 0.2 M acetic acid, 0.5 M NaCl) to remove bound, but not internalized, EGF or DAB<sub>389</sub>EGF. EGF binding capacity was measured by incubating the cells, on ice, with [<sup>125</sup>I]EGF. Following a 90-minute incubation the cells were washed, solubilized, and counted.

An EGF receptor-dependent interaction is also shown by the fact that DAB<sub>389</sub>EGF, like EGF, displaces [<sup>125</sup>I]EGF from the EGF receptor, as shown in Fig. 11. In Fig. 11 open squares indicate EGF and solid diamonds indicate DAB<sub>389</sub>EGF. Results in Fig. 11 are expressed as a percent of control (no competition) cpm. The ability of DAB<sub>389</sub>EGF to displace high affinity [<sup>125</sup>I]EGF binding to A431 cells was evaluated as follows. A431 cells, plated in triplicate wells of 24 well plates, were preincubated in binding media (phosphate buffered saline pH 7.2 + 0.1% BSA + 15 mM sodium azide + 50 mM 2-deoxyglucose) for 1 hour at 37°C and then incubated with [<sup>125</sup>I]EGF in binding media in the presence of DAB<sub>389</sub>EGF or EGF. Following incubation, the cells were washed, solubilized and counted. The results are summarized in Table 4.

In Table 4 EC<sub>50</sub> is the concentration resulting in displacement of 50% of the [<sup>125</sup>I] EGF.

Table 4: Displacement of [<sup>125</sup>I] EGF Binding by EGF and DAB<sub>389</sub>EGF

Competition	EC <sub>50</sub>	fold over [ <sup>125</sup> I]EGF	fold over EGF
EGF	1.0 x 10 <sup>-8</sup> M	20	-
DAB <sub>389</sub> EGF	4.5 x 10 <sup>-7</sup> M	900	45

Cytotoxicity of DT-EGF Chimeric Toxins is DT Dependent.

Upon binding to its receptor, the cellular uptake of native DT occurs by endocytosis of clathrin coated vesicles (Middlebrook et al. (1978) J. Biol. Chem. 253:7325). DT is then found in endosomes where the low pH induces a conformational change facilitating the translocation of the enzymatic fragment A portion of DT into the cytosol. To determine if the cytotoxicity of DAB<sub>486</sub>EGF and DAB<sub>389</sub>EGF is also dependent upon the same pathway, A431 cells were plated in sextuplicate wells of 96 well plates containing DAB<sub>486</sub>EGF, DAB<sub>389</sub>EGF or DMEM + 10% FCS in the absence or presence of chloroquine (10<sup>-5</sup> M) (Sigma). Chloroquine is a lysosomotropic compound which prevents acidification of endosomes (Kim et al. (1965) J. Bacteriol. 90:1552). Following a 20-hour incubation at 37°C, the cells were labeled with [<sup>3</sup>H]leucine, trypsinized, harvested onto glass fiber filter mats and counted. The results are shown in Table 5, expressed as the percent of control (no DAB<sub>486</sub>EGF or DAB<sub>389</sub>EGF) incorporation and represent the mean of three experiments. The results show that chloroquine blocks the cytotoxicity of DT-EGF chimeric toxins.

Table 5: Sensitivity of DAB-EGF Chimeric Toxin-Cytotoxicity to Chloroquine

Percent of Control Incorporation			
	DAB <sub>486</sub> EGF Concentration	No Addition	Chloroquine
5	0	100	86
	10 <sup>-8</sup> M	5	60
	10 <sup>-9</sup> M	25	96
10	DAB <sub>389</sub> EGF Concentration		
	0	100	73
	10 <sup>-11</sup> M	4	61
15	10 <sup>-12</sup> M	57	100

Following translocation into the cytosol, fragment A catalyzes the cleavage of NAD and the covalent linkage of ADP-ribose to elongation factor 2 (EF-2) resulting in the inhibition of protein synthesis (Bacha et al. (1983) J. Biol. Chem. 258:1565). To evaluate the mechanism by which DAB<sub>486</sub>EGF inhibits protein synthesis, A431 cells were plated in triplicate wells of 24 well plates containing DT, DAB<sub>486</sub>EGF, or complete medium. Following a 20-hour incubation at 37°C, the cells were washed and incubated in lysis buffer (10mM Tris, 10mM NaCl, 3mM Mg Cl<sub>2</sub>, 10mM thymidine, 1mM EGTA, 1% TRITON X-100) with [<sup>32</sup>P]NAD in the presence of purified DT fragment A (Calbiochem). TCA precipitable extracts were collected on glass fiber filters and counted to quantitate the percent of control EF-2 available for ADP-ribosylation. The results of these experiments are shown in Table 6. DAB<sub>486</sub>EGF, like DT, reduced (in a dosage dependent manner) the amount of EF-2 available for ADP ribosylation.



Table 6: ADP-Ribosylation of EF-2 by DAB<sub>486</sub>EGF

Toxin	Concentration	Percent of Control Level of EF-2 Available for ADP-ribosylation
DT	10 <sup>-8</sup> M	<1
	10 <sup>-9</sup> M	17
DAB <sub>486</sub> EGF	10 <sup>-8</sup> M	13
	10 <sup>-9</sup> M	20

DAB<sub>389</sub>EGF Is An Improved Chimeric Toxin.

DAB<sub>389</sub>EGF is far more toxic than is DAB<sub>486</sub>EGF. As shown in Figs. 8 and 9, DAB<sub>389</sub>EGF exhibits an IC<sub>50</sub> for the inhibition of protein synthesis in A431 cells approximately 10 times lower than that of DAB<sub>486</sub>EGF (DAB<sub>389</sub>EGF IC<sub>50</sub> = 3 x 10<sup>-13</sup> M; DAB<sub>486</sub>EGF IC<sub>50</sub> = 3 x 10<sup>-12</sup> M).

The greater potency of DAB<sub>389</sub>EGF is also shown in experiments measuring the rapidity with which DAB<sub>389</sub>EGF and DAB<sub>486</sub>EGF kill A431 cells. Figs. 12 and 12 show the exposure time (of A431 cells to DAB<sub>486</sub>EGF or DAB<sub>389</sub>EGF) required to induce maximal inhibition of protein synthesis. Cells were exposed to DAB<sub>486</sub>EGF (5 x 10<sup>-9</sup> M) (Fig. 12) or DAB<sub>389</sub>EGF (5 x 10<sup>-9</sup> M) (Fig. 13) for the indicated times and then washed of unbound DAB<sub>486</sub>EGF or DAB<sub>389</sub>EGF. Following an overnight incubation in complete media (DMEM + 10% FCS), the cells were labeled with [<sup>14</sup>C]leucine. The results show that near maximal inhibition of protein synthesis occurs following a 15-minute exposure to

DAB<sub>389</sub>EGF while a greater than 75-minute exposure is required for DAB<sub>486</sub>EGF.

The kinetics of protein synthesis inhibition in DAB<sub>389</sub>EGF or DAB<sub>486</sub>EGF treated A431 cells is shown in Fig. 14. To examine the kinetics of protein synthesis inhibition A431 cells were incubated with DAB<sub>486</sub>EGF ( $5 \times 10^{-9}$ ) or DAB<sub>389</sub>EGF ( $5 \times 10^{-9}$  M) in complete medium at 37°C. At the indicated times, DAB<sub>486</sub>EGF or DAB<sub>389</sub>EGF was removed and the cells were labeled with [<sup>14</sup>C]leucine for 1 hour. The results indicate that there is a 50% reduction in protein synthesis following a 1-hour incubation with DAB<sub>389</sub>EGF while maximal inhibition occurs by 4 hours. Maximal inhibition of protein synthesis occurs more than 6 hours following incubation with DAB<sub>486</sub>EGF.

Use

The improved chimeric toxins of the invention are administered to a mammal, e.g., a human, suffering from a medical disorder, e.g., cancer, or other conditions characterized by the presence of a class of unwanted cells to which a polypeptide ligand can selectively bind. The amount of protein administered will vary with the type of disease, extensiveness of the disease, and size of species of the mammal suffering from the disease. Generally, amounts will be in the range of those used for other cytotoxic agents used in the treatment of cancer, although in certain instances lower amounts will be needed because of the specificity and increased toxicity of the improved chimeric toxins.

The improved chimeric toxins can be administered using any conventional method; e.g., via injection, or via a timed-release implant. In the case of MSH improved chimeric toxins, topical creams can be used to kill primary cancer cells, and injections or implants

can be used to kill metastatic cells. The improved chimeric toxins can be combined with any non-toxic, pharmaceutically-acceptable carrier substance.

#### Other Embodiments

5 Other embodiments are within the following claims. For example, chimeric toxins have been constructed, by methods known to those skilled in the art, in which DAB<sub>389</sub> and DAB<sub>486</sub> have been fused to interleukin 4 (IL-4). DAB<sub>389</sub>-IL-4 is about 10 times  
10 more cytotoxic than is DAB<sub>486</sub>-IL-4. DAB<sub>389</sub> has also been fused to interleukin 6. DAB<sub>486</sub> and DAB<sub>389</sub> have also been fused to human chorionic gonadotropin. The improved chimeric toxins of the invention include portions of DT fused to any cell-specific polypeptide  
15 ligand which has a binding domain specific for the particular class of cells which are to be intoxicated. Polypeptide hormones are useful such ligands. Chimeric toxins, e.g., those made using the binding domain of  $\alpha$  or  $\beta$  MSH, can selectively bind to melanocytes, allowing  
20 the construction of improved DT-MSH chimeric toxins useful in the treatment of melanoma. Other specific-binding ligands which can be used include insulin, somatostatin, interleukins I and III, and granulocyte colony stimulating factor. Other useful  
25 polypeptide ligands having cell-specific binding domains are follicle stimulating hormone (specific for ovarian cells), luteinizing hormone (specific for ovarian cells), thyroid stimulating hormone (specific for thyroid cells), vasopressin (specific for uterine cells,  
30 as well as bladder and intestinal cells), prolactin (specific for breast cells), and growth hormone (specific for certain bone cells). Improved chimeric toxins using these ligands are useful in treating

cancers or other diseases of the cell type to which there is specific binding.

For a number of cell-specific ligands, the region within each such ligand in which the binding domain is located is now known. Furthermore, recent advances in solid phase polypeptide synthesis enable those skilled in this technology to determine the binding domain of practically any such ligand, by synthesizing various fragments of the ligand and testing them for the ability to bind to the class of cells to be labeled. Thus, the chimeric toxins of the invention need not include an entire ligand, but rather may include only a fragment of a ligand which exhibits the desired cell-binding capacity. Likewise, analogs of the ligand or its cell-binding region having minor sequence variations may be synthesized, tested for their ability to bind to cells, and incorporated into the hybrid molecules of the invention. Other potential ligands include monoclonal antibodies or antigen-binding, single-chain analogs of monoclonal antibodies, where the antigen is a receptor or other moiety expressed on the surface of the target cell membrane.

What is claimed is:

1           1. A chimeric toxin comprising protein  
2 fragments joined together by peptide bonds, said  
3 chimeric toxin comprising, in sequential order,  
4 beginning at the amino terminal end of said chimeric  
5 toxin,

6           (a) the enzymatically active Fragment A of  
7 diphtheria toxin,

8           (b) a first fragment including the cleavage  
9 domain  $1_1$  adjacent said Fragment A of diphtheria toxin,

10          (c) a second fragment comprising at least a  
11 portion of the hydrophobic transmembrane region of  
12 Fragment B of diphtheria toxin, said second fragment  
13 having a deletion of at least 50 diphtheria toxin amino  
14 acid residues, said deletion being C-terminal to said  
15 portion of the transmembrane region, and said second  
16 fragment not including domain  $1_2$ , and

17          (d) a third fragment comprising a portion of a  
18 cell-specific polypeptide ligand, said portion including  
19 at least a portion of the binding domain of said  
20 polypeptide ligand, said portion of said binding domain  
21 being effective to cause said chimeric toxin to bind  
22 selectively to a predetermined class of cells to be  
23 attacked by said enzymatically active Fragment A, said  
24 chimeric toxin possessing any of, greater toxicity than  
25 that of a toxin comprised of  $DAB_{486}$  fused to said  
26 third fragment, a lower  $K_d$  for the sites on cells of  
27 said predetermined class to which said chimeric toxin  
28 binds than that of a toxin comprised of  $DAB_{486}$  fused  
29 to said third fragment, greater resistance to  
30 proteolytic degradation than that exhibited by a toxin  
31 comprised of  $DAB_{486}$  fused to said third fragment,  
32 greater resistance to the inhibition of its cytotoxicity  
33 by said cell-specific polypeptide ligand than that  
34 exhibited by  $DAB_{486}$  fused to said third fragment, the

35 ability to inhibit protein synthesis to a given degree  
36 by a period of exposure that is shorter than the period  
37 of exposure required by DAB<sub>489</sub> fused to said third  
38 fragment to inhibit protein synthesis to the same  
39 degree, or the ability to effect a more rapid onset of  
40 the inhibition of protein synthesis than that exhibited  
41 by DAB<sub>486</sub> fused to said third fragment.

1           2. The chimeric toxin of claim 1, wherein  
2 said deletion is at least 80 diphtheria toxin amino acid  
3 residues in length.

1           3. The chimeric toxin of claim 1, wherein  
2 said Fragment B of diphtheria toxin does not include any  
3 diphtheria toxin sequences C-terminal to amino acid  
4 residue 386 of native diphtheria toxin.

1           4. The chimeric toxins of claim 1, wherein  
2 (a), (b), and (c) comprise DAB<sub>389</sub>.

1           5. The chimeric toxin of claim 1, wherein  
2 said portion of said polypeptide ligand is a portion of  
3 epidermal growth factor effective to cause said chimeric  
4 toxin to bind to cells bearing the epidermal growth  
5 factor receptor.

1           6. The chimeric toxin of claim 5, wherein (a),  
2 (b), and (c) comprise DAB<sub>389</sub> and (d) comprises EGF.

1           7. The chimeric toxin of claim 5, wherein said  
2 chimeric toxin possessing any of, greater toxicity than  
3 that of a toxin comprised of DAB<sub>486</sub> fused to EGF, a  
4 lower  $K_d$  for the sites on cells of said predetermined  
5 class to which said chimeric toxin binds than that of a

6 toxin comprised of DAB<sub>486</sub> fused to EGF, greater  
7 resistance to the inhibition of its cytotoxicity by EGF  
8 than that exhibited by DAB<sub>486</sub> fused to EGF, the  
9 ability to inhibit protein synthesis to a given degree  
10 by a period of exposure that is shorter than the period  
11 of exposure required by DAB<sub>489</sub> fused to EGF to inhibit  
12 protein synthesis to the same degree, or the ability to  
13 effect a more rapid onset of the inhibition of protein  
14 synthesis than that exhibited by DAB<sub>486</sub> fused to EGF.

1 8. The chimeric toxin of claim 1, wherein said  
2 portion of said polypeptide ligand is a portion of  
3 interleukin 2 effective to cause said chimeric toxin to  
4 bind to T cells.

1 9. The chimeric toxin of claim 8, wherein (a),  
2 (b), and (c) comprise DAB<sub>389</sub> and (d) comprises amino  
3 acid residues 2-133 of IL-2.

1 10. The chimeric toxin of claim 8, wherein  
2 said chimeric toxin possessing any of, greater toxicity  
3 than that of a toxin comprised of DAB<sub>486</sub> fused amino  
4 acid residues 2-133 of IL-2, a lower K<sub>d</sub> for the sites  
5 on cells of said predetermined class to which said  
6 chimeric toxin binds than that of a toxin comprised of  
7 DAB<sub>486</sub> fused to amino acid residues 2-133 of IL-2, or  
8 greater resistance to proteolytic degradation than that  
9 exhibited by a toxin comprised of DAB<sub>486</sub> fused to  
10 amino acid residues 2-133 of IL-2.

1 11. The chimeric toxin of claim 1, wherein  
2 said chimeric toxin is encoded by a fused gene  
3 comprising regions coding for said protein fragments.

1                   12. A DNA sequence encoding the chimeric toxin  
2 of claim 1.

1                   13. An expression vector containing the DNA  
2 sequence of claim 12.

1                   14. A cell transformed with the vector of  
2 claim 13.

1                   15. A method of producing the chimeric toxin  
2 of claim 1 comprising culturing the cell of claim 14,  
3 and isolating said chimeric toxin from said cultured  
4 cell or supernatant.

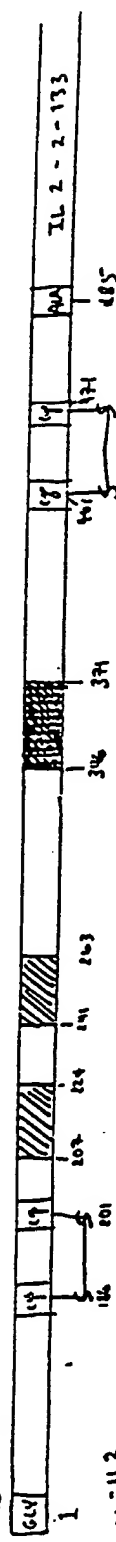


FIG 1

(a)



(b) DAB<sub>465</sub>-IL-2



DAB<sub>465</sub>-IL-2



DAB<sub>465</sub>-IL-2



DAB<sub>389</sub>-IL-2



DAB<sub>295</sub>-IL-2



DAB<sub>(Δ205-289)</sub>-IL-2



DAB<sub>(Δ205-289)</sub><sub>334</sub>-IL-2



14

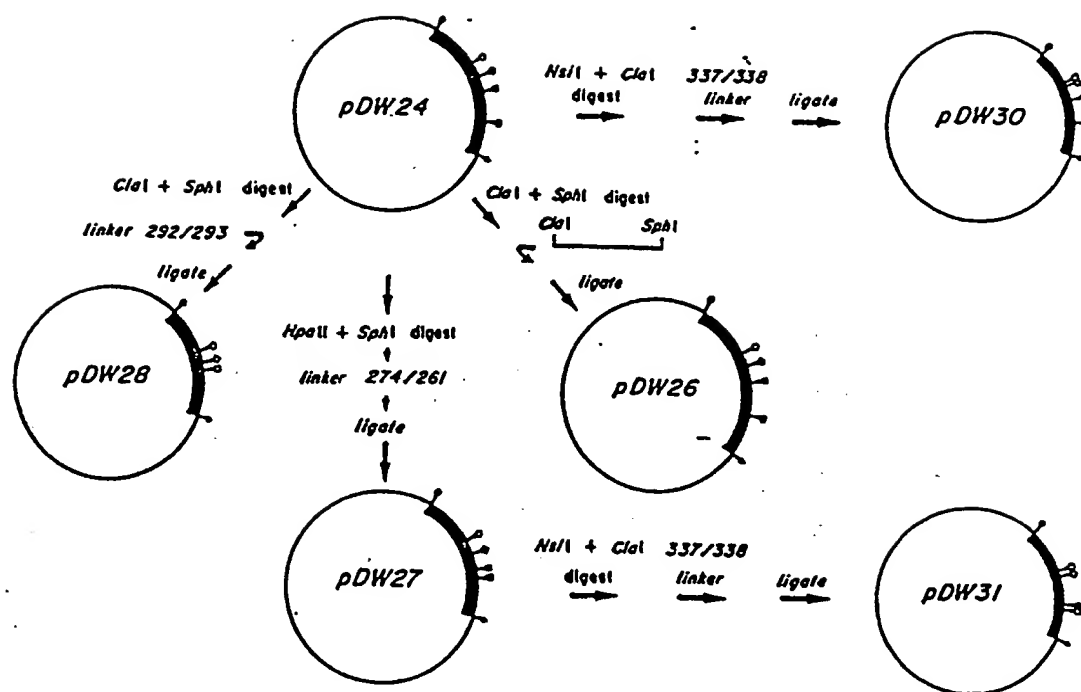


FIG 2

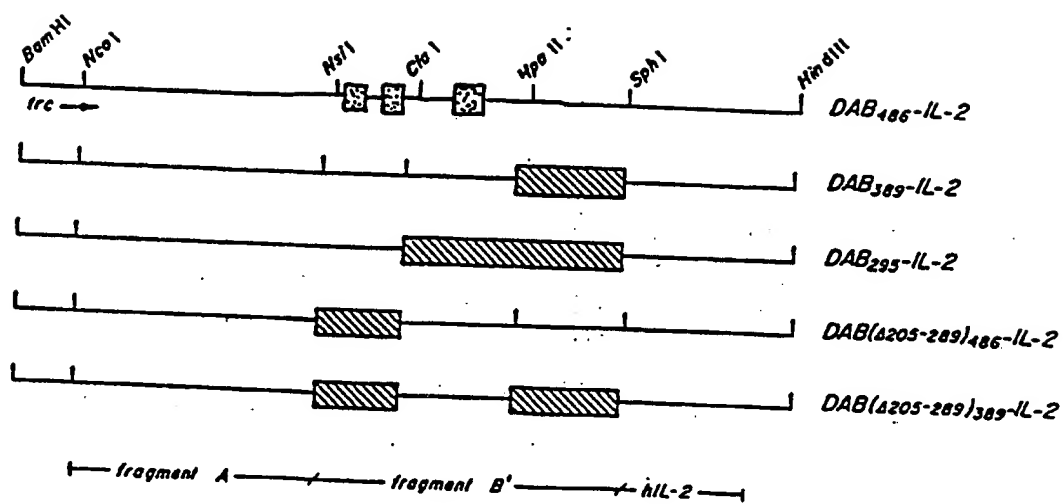
3/  
14

FIG 3

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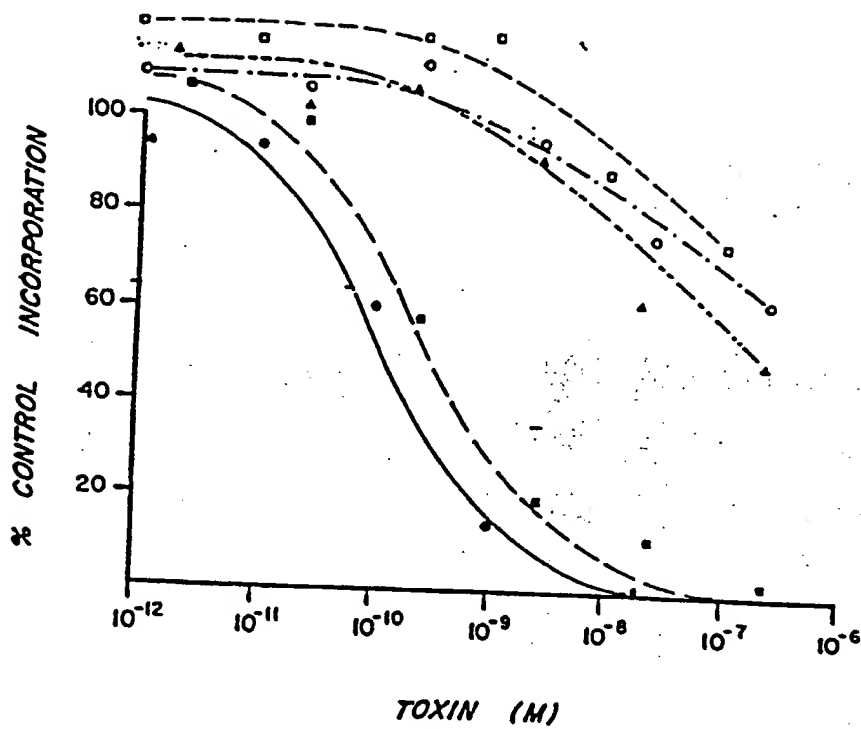


FIG 4

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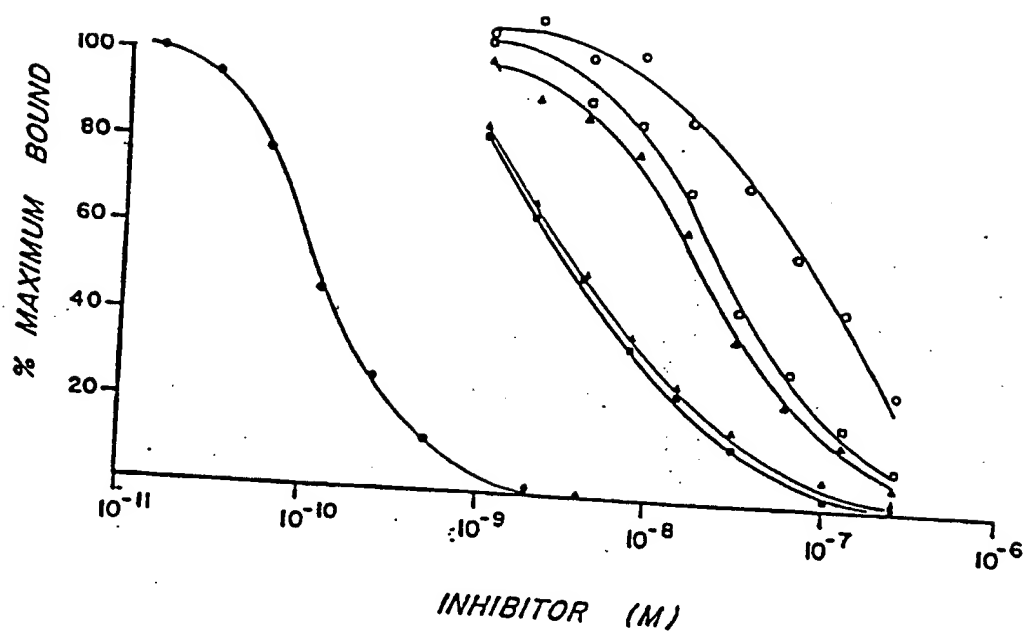


FIG. 5

FIG. 6: THE SYNTHETIC EGF SEGMENT

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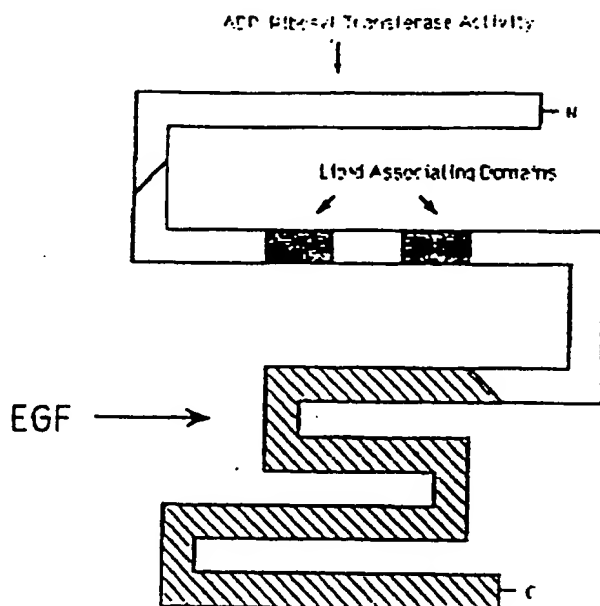
<u>SphI</u>	Asn	Ser	Asp	Ser	Glu	Cys	Pro	Leu	Ser	His	Asp	Gly
GCATGCT	AAC	AGC	GAC	AGC	GAA	TGT	CCG	CTG	AGC	CAC	GAC	GGT
<u>CGTACGA</u>	TTG	TCG	CTG	TCG	CTT	ACA	GGC	GAC	TCG	GTG	CTG	CCA
Tyc	Cys	Leu	His	Asp	Gly	Val	Cys	Het	Tyr	Ile	Glu	Ala
TAC	TGT	CTG	CAC	GAC	GGT	GTT	TGT	ATG	TAC	ATC	GAA	GCT
ATG	ACA	GAC	GTG	CTG	CCA	CAA	ACA	TAC	ATG	TAG	CTT	CGA
Leu	Asp	Lys	Tyr	Ala	Cys	Asn	Cys	Val	Val	Gly	Tyr	Ile
CTA	GAC	AAA	TAC	GCT	TGT	AAC	TGT	GTT	GTT	GGT	TAC	ATC
GAT	CTG	TTT	ATG	CGA	ACA	TTG	ACA	CAA	CAA	CCA	ATG	TAG
Gly	Glu	Arg	Cys	Gln	Tyr	Arg	Asp	Leu	Lys	Trp	Trp	Glu
GGT	GAA	CGC	TGT	CAG	TAC	CGC	GAC	CTG	AAA	TGG	TGG	GAA
CCA	CTT	GCG	ACA	GTC	ATG	GCG	CTG	GAC	TTT	ACC	ACC	CTT
Leu	Arg	<u>STOP</u>										
CTG	CGC	TGAAGTACTAATTTACGTACCGGAGGCCTAAGGAGCCC										
GAC	GCG	ACTTCATGATTAAATGCATGGCCTCCGGATTCTCGGG										

TrpA Terminator
HindIII  
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 CGGATTACTCGCCCGAAAAAAGGCAGCTGTTCCGGACTTGCAGCTTCGAA

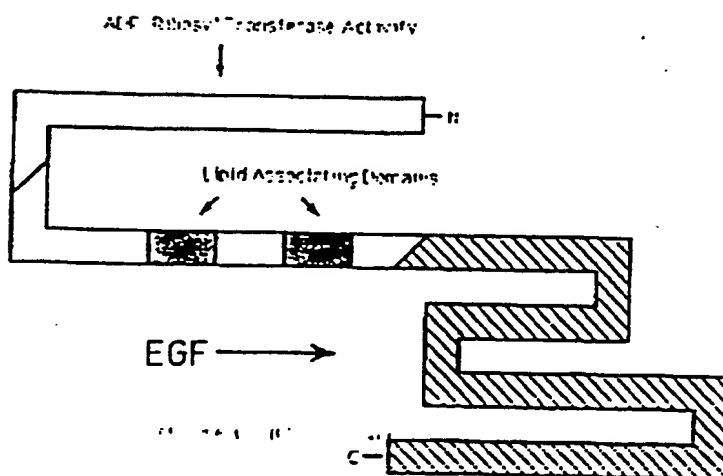
APPL0554

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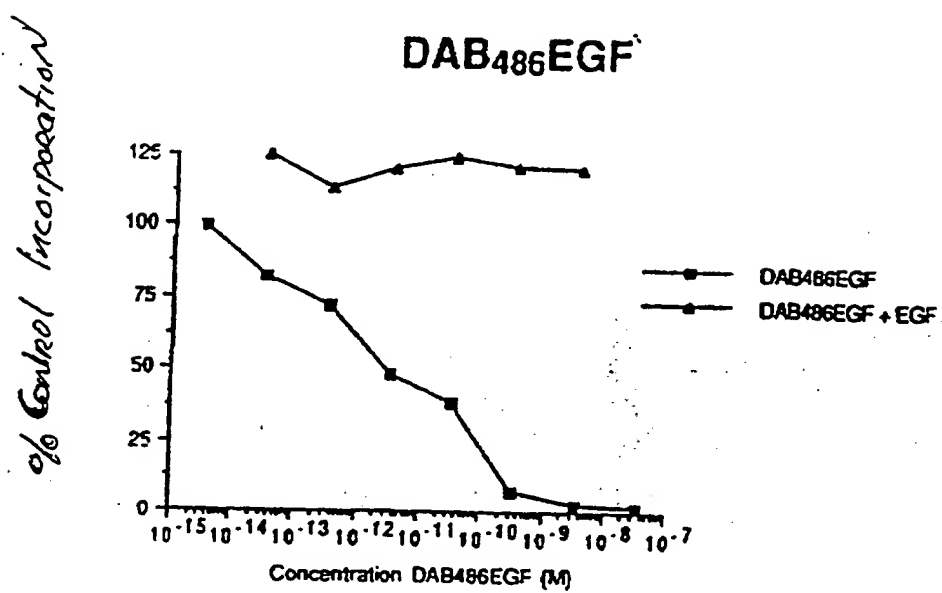
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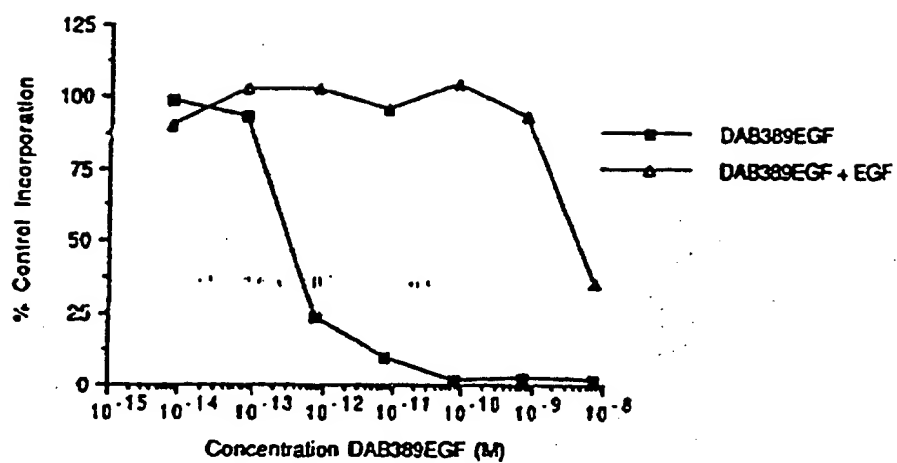


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**DAB<sub>389</sub>EGF**

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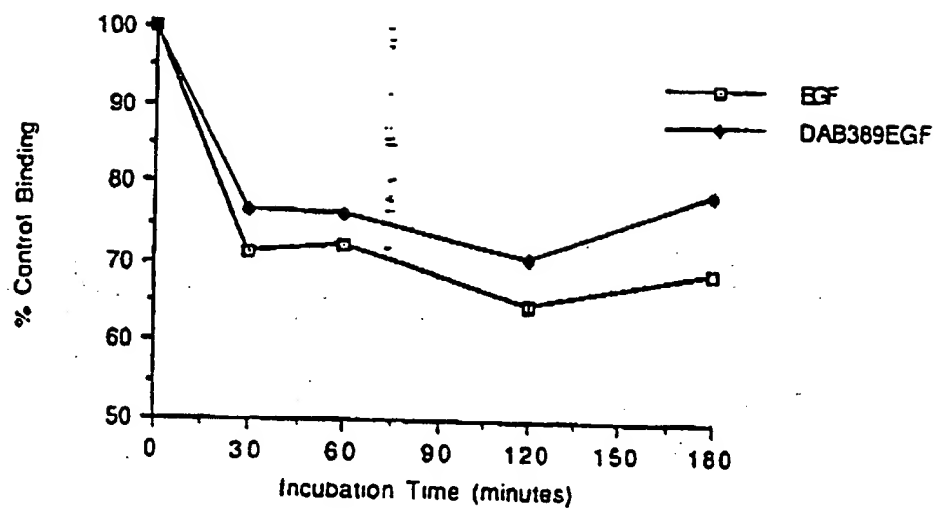


Fig 10

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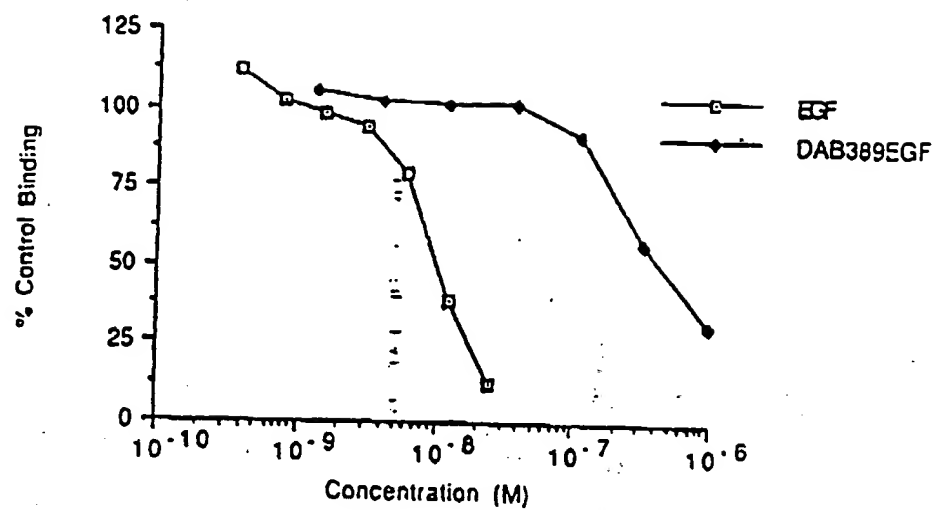
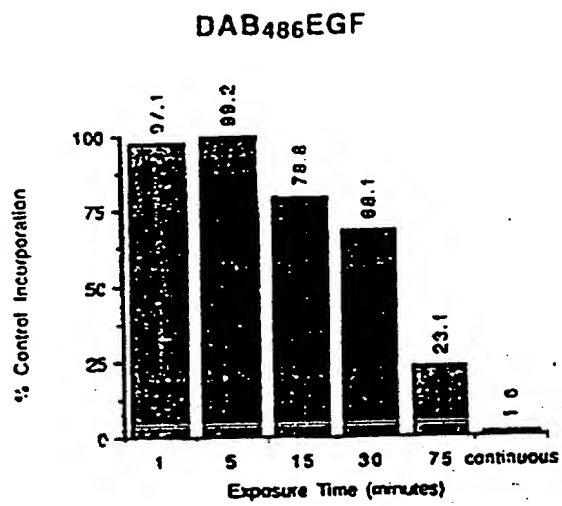


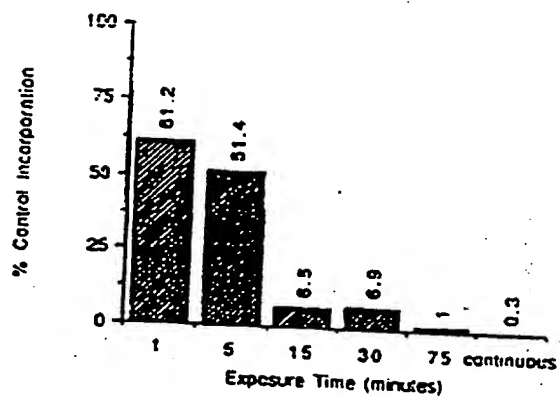
FIG 11

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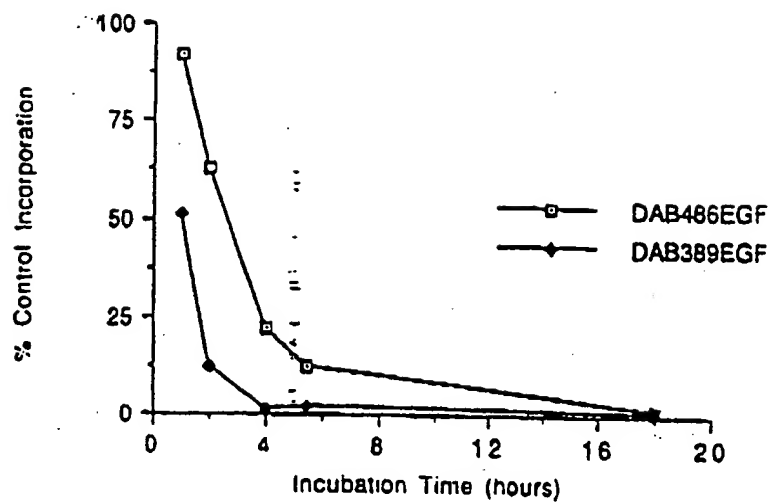


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**DAB<sub>389</sub>EGF**



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# INTERNATIONAL SEARCH REPORT

International Applicant No. **PCT/US91/01282**

**I. CLASSIFICATION OF SUBJECT MATTER** (In several classification symbols apply, indicate all) \*

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C 07K 7/10, 13/00

US CL.: 530/350, 324, 402; 435/69.7, 194

**II. FIELDS SEARCHED**

Minimum Documentation Searched <sup>7</sup>

Classification System	Classification Symbols
U.S. CL. . .	530/350, 324, 402; 435/69.7, 194

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched \*

DIALOG

**III. DOCUMENTS CONSIDERED TO BE RELEVANT \***

Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
Y	US.A. 4.830.962 (Gelfand et al.) 16 May 1989. see entire document.	1-11,15
X Y	US.A. 4.675.382 (Murphy et al.) 23 June 1987. see entire document.	1-11,15 1-11,15
Y	Protein Engineering, volume 1, issued 1987. Williams et al.. "Diphtheria toxin receptor binding domain substitution with interleukin-2: genetic construction and properties of a diphtheria toxin-related interleukin-2 fusion protein". pages 493-498. see entire document.	1-11,15
Y	Journal of Experimental Medicine, volume 167, issued February 1988. Bacha et al.. "Interleukin-2 receptor-targeted cytotoxicity", pages 612-622. see entire document.	1-11,15

\* Special categories of cited documents: <sup>10</sup>

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claims) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"Z" document member of the same patent family

**IV. CERTIFICATION**

Date of the Actual Completion of the International Search

Date of Making of this International Search Report

23 MAY 1991

12 JUN 1991

International Searching Authority

Signature of Author and Official

ISA/US

K. L. HENDRICKS

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Journal of Biological Chemistry. Volume 264, No 9, issued 25 March 1989. Cabiliaux et al., "Secondary Structure of Diphtheria Toxin and its Fragments Interacting with Acidic Liposomes Studied by Polarized Infrared Spectroscopy", pages 4928-4938, see entire document	1-11,15
Y	Journal of Biological Chemistry, volume 258, No.3, issued 10 February 1983, Bacha et al., "Thyrotropin-releasing Hormone-Diphtheria Toxin-related Polypeptide Conjugates", pages 1565-1570, see entire document.	1-11,15
Y	Journal of Biological Chemistry, volume 261, No.7, Issued 05 March 1986, Colombatti et al., "Cloned Fragment of Diphtheria Toxin linked to T cell-specific Antibody Identifies Regions of B chain Active in Cell Entry", pages 3030-3035, see entire document	1-11,15



## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers \_\_\_\_\_, because they relate to subject matter <sup>12</sup> not required to be searched by this Authority, namely:
  
2. ☐ Claim numbers \_\_\_\_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out <sup>13</sup>, specifically:
  
3. ☐ Claim numbers \_\_\_\_\_, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

Invention I: claims 1-11 and 15, a chimeric toxin and method of production.

Invention II: claims 12-14, DNA, vector, and cell.

Groups I and II do not meet the requirements for unity of Invention, as they are materially distinct products.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
  
3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:  
claims 1-11, 15
4. ☐ As all searchable claims could be searched without effort, notwithstanding an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remarks on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ The protest accompanied the payment of additional search fees.